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[29] Dynamics and Thermodynamics of Hyperthermophilic Proteins by Hydrogen Exchange

By S. WALTER ENGLANDER and REUBEN HILLER

Introduction

What strategies do hyperthermophilic proteins use to maintain their stability and activity at high temperature? The measurement of stability at very high temperatures and possibly correlated characteristics such as dynamic flexibility present special problems. Here we consider the application of hydrogen exchange approaches to these problems. Thermodynamic parameters and aspects of structural dynamics can, in many cases, be measured by hydrogen exchange methods at temperatures far below the melting temperature.

Thermal Stability

It is often assumed that the unusually high melting temperatures of thermophilic proteins reflect an unusually large stability, measured by the free energy for global unfolding. However, high melting temperature can be achieved in other ways as well. The stabilization free energy of a protein as a function of temperature, $\Delta G(T)$, is described by the Gibbs–Helmholtz equation [Eq. (1)].

$$\Delta G(T) = \Delta H_m + \Delta C_p(T - T_m) - T(\Delta S_m + \Delta C_p \ln T/T_m) \qquad (1)$$

Alternative curves that fit Eq. (1) are shown in Fig. 1. The melting temperature, T_m, occurs where the difference in free energy between the native and unfolded states passes through zero. Figure 1 and Eq. (1) show that high melting temperature can be achieved in several different ways. The addition of stabilizing interactions will increase $\Delta G(T)$, shifting the melting curve vertically upward and increasing T_m. High T_m can also be achieved by a lateral shift in the free energy curve to higher temperature. This requires a change in either the melting enthalpy (ΔH_m) or entropy (ΔS_m), or in both. High T_m can also be achieved by flattening the curve so that it crosses the ΔG equals zero position at higher (and lower) temperature.

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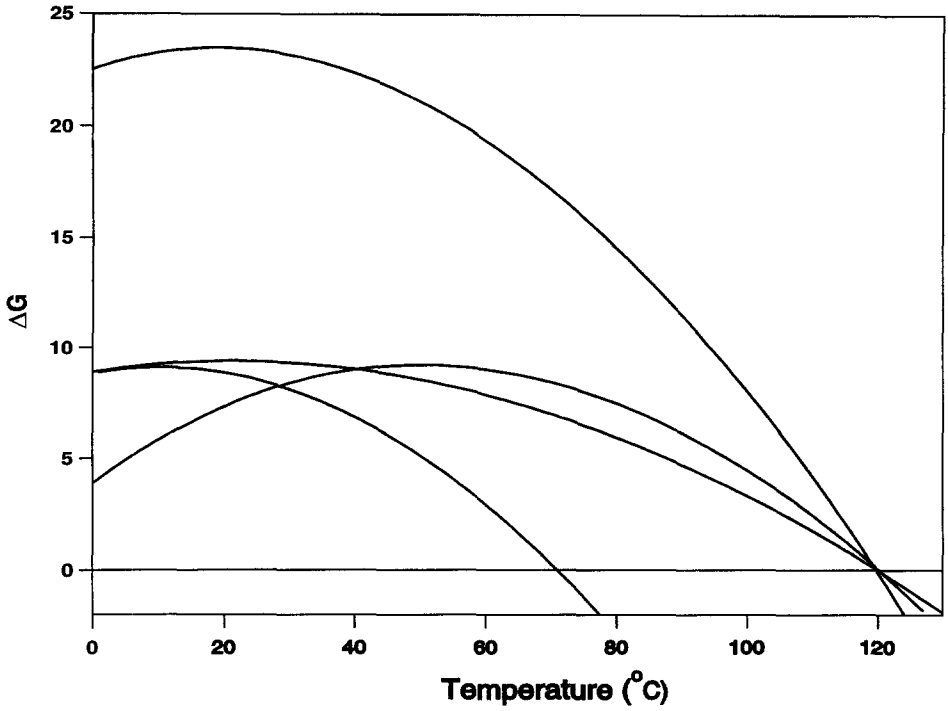

FIG. 1. Alternative thermal stability curves that yield high melting temperature. Mesophilic behavior (T_m of 76°) can be readjusted to higher T_m (120° shown) by altering the different parameters in Eq. (1) to raise, shift, or stretch the stability curve.

This requires changing the balance of hydrophobic interactions to decrease the specific heat parameter (ΔC_p).

For mesophilic proteins, one classically determines stability parameters and mutationally induced changes in stability by driving the protein through its thermal transition where the melting behavior can be measured.[1-3] The parameters determined can then be used together with Eq. (1) to infer the shape and positioning of the stability curve. The high T_m values of thermophilic proteins make these measurements problematic and impose additional uncertainty on the long extrapolations that this analysis requires. When melting is not fully reversible, as often occurs, these approaches fail.

Hydrogen exchange (HX) methods now make it possible to measure the stability parameters of native proteins, the distribution of stability and structural

[1] P. L. Privalov, *Adv. Protein Chem.* **33,** 167 (1979).
[2] P. L. Privalov, *Adv. Protein Chem.* **35,** 1 (1982).
[3] C. N. Pace, *CRC Crit. Rev. Biochem.* **3,** 1 (1975).

dynamics through the protein, and changes in these parameters due to experimentally imposed mutations, both locally and remotely.

Hydrogen Exchange and Protein Unfolding

Hydrogens on the polar groups of proteins engage in continual exchange with hydrogens in the aqueous solvent. These groups include the ionizable and neutral polar side chains and also the main chain peptide group NH hydrogen. Slowly exchanging protein hydrogens largely represent the main chain amide groups that participate extensively in secondary and tertiary structural H-bonding. Amide HX is rate-limited by a proton transfer reaction that requires H-bond formation to a solvent hydroxide ion (above pH 3). Because H-bonded NHs are sterically inaccessible to this process, their exchange requires transient H-bond breakage.

Hydrogen bonds can be broken in various kinds of opening reactions. These include small local fluctuations that break one H bond at a time, subglobal unfolding reactions (local unfolding) that break multiple H bonds in a cooperative partial unfolding reaction, and large whole-molecule global unfolding reactions. Thermodynamic principles require that protein molecules continually cycle through all of these high energy conformational states and that they populate each state according to the Boltzmann relationship in Eq. (2).

$$\Delta G_{op} = -RT \ln K_{op} \tag{2}$$

ΔG_{op} is the free energy of any given state (I or U) above the native state and K_{op} is the equilibrium constant for unfolding, given by I/N or U/N. In many cases, the most slowly exchanging hydrogens in a protein are so well protected that they can only exchange during the small fraction of time when the protein visits the globally unfolded state. The measurement of their exchange rates can directly evaluate the free energy for global unfolding under fully native conditions.[4,5] The faster exchange of hydrogens that are exposed by smaller opening reactions can provide structurally resolved information on local dynamics and energetics through the protein. HX experiments can be manipulated to distinguish and measure the different opening reactions.

Hydrogen Exchange Measurement and Analysis

HX has been measured by all combinations of H–H, H–D, and H–T approaches. At lowest resolution, HX can be measured at a whole molecule level using radioisotopic tritium counting[6] and by various spectroscopic methods that are sensitive

[4] S. N. Loh, K. E. Prehoda, J. Wang, and J. L. Markley, *Biochemistry* **32,** 11022 (1993).
[5] Y. Bai, J. S. Milne, L. Mayne, and S. W. Englander, *Proteins: Struct. Funct. Genet.* **20,** 4 (1994).
[6] S. W. Englander and J. J. Englander, *Methods in Enzymology* **49,** 24 (1978).

to H–D differences, including infrared[7] and ultraviolet[8] absorbance and Raman scattering.[9] At intermediate resolution, HX can be measured by tritium exchange labeling together with fragment separation methods,[10] and this approach has been extended to mass spectroscopic approaches.[11]

Most effectively, HX can be measured at the level of amino acid resolution by NMR spectroscopy using standard two-dimensional (2D) methods for relatively small protein molecules (<25 kDa). The NMR machine-time requirement can be reduced and faster HX rates can be measured by using ^{15}N-labeled protein together with heteronuclear nuclear magnetic resonance (NMR) methods, typically the heteronuclear single quantum COSY experiment. Alternatively, straightforward 1D proton NMR can be used, especially when only the slowest hydrogens are to be studied, since the prior exchange of the many faster hydrogens with deuterium simplifies the spectrum and can allow resolution of individual slowly exchanging NHs. This approach can even allow quite large proteins to be studied, especially for thermophilic proteins where the NMR measurements can be done at high temperatures that enhance molecular tumbling and sharpen NMR lines. Analogously, faster sites can be better resolved by using partially exchanged proteins prepared with the slower sites already deuterated.

HX can be initiated by placing a protein into D_2O under the conditions to be studied (pD, temperature, denaturant, etc.). Each hydrogen then begins to exchange with solvent deuterium at its characteristic rate. The exchange process can be followed by recording sequential NMR spectra in time. The measured first-order exchange rate (k_{ex}) can then be compared with the chemical rate expected for that site in the fully exposed condition (k_{ch}[cat]) in order to obtain the structural protection factor ($P = 1/K_{op}$) (where [cat] is the HX catalyst concentration, namely OH^- for amide NHs above pH 3). From this the free energy for the dominant opening reaction that exposes the site to exchange can be computed [Eq. (3)].

$$\Delta G_{HX} = -RT \ln k_{ex}/k_{ch}[cat] = -RT \ln 1/P = -RT \ln K_{op} \qquad (3)$$

Equation (3) assumes that exchange occurs only in the open state with the externally calibrated chemical rate constant, k_{ch}. The k_{ch} value depends on pH, temperature, local amino acid sequence, and other more minor factors[12,13] and can most easily be computed using spreadsheets that have been placed on the World Wide Web. When the measurement is not done at amino acid resolution, some averaged k_{ch} value might be used (e.g., characteristic for the Ala-NH-Ala proton).

[7] H. H De Jongh, E. Goormaghtigh, and J. M. Ruysschaert, *Biochemistry* **34,** 172 (1995).
[8] J. J. Englander, D. B. Calhoun, and S. W. Englander, *Anal. Biochem.* **92,** 517 (1979).
[9] P. Hildebrandt, F. Vanhacke, G. Heibel, and A. G. Mauk, *Biochemistry* **32,** 14158 (1993).
[10] J. J. Englander, J. R. Rogero, and S. W. Englander, *Anal. Biochem.* **147,** 234 (1985).
[11] Z. Zhang, C. B. Post, and D. L. Smith, *Biochemistry* **35,** 779 (1996).
[12] G. P. Connelly, Y. Bai, M.-F. Jeng, L. Mayne, and S. W. Englander, *Proteins: Struct. Funct. Genet.* **17,** 87 (1993).
[13] Y. Bai, J. S. Milne, L. Mayne, and S. W. Englander, *Proteins: Struct. Funct. Genet.* **17,** 75 (1993).

An important structural issue concerns the relationship between k_{ch} and opening–closing rates. The use of Eq. (3) assumes the relationship in Eq. (4).

$$k_{ex} = K_{op}\, k_{ch}[\text{cat}] \tag{4}$$

A more complete formulation uses Eq. (5).

$$k_{ex} = k_{ch}[\text{cat}]\, k_{op}/(k_{op} + k_{cl} + k_{ch}[\text{cat}]) \tag{5}$$

For stable structure, $k_{op} \ll k_{cl}$ so the k_{op} term in the denominator can be safely ignored. When $k_{cl} \gg k_{ch}[\text{cat}]$, the denominator in Eq. (5) then reduces to Eq. (4). Exchange then depends only on the preequilibrium opening and is said to be in the EX2 limit,[14] meaning that exchange follows second-order kinetics dependent on solvent catalyst concentration. When reclosing is slower than $k_{ch}[\text{cat}]$, HX is in the EX1 limit (monomolecular exchange) and yields the first-order opening rate constant ($k_{ex} = k_{op}$) rather than the opening equilibrium constant. A thorough discussion of these and related issues is given in ref. 15.

HX due to local fluctuations is unavoidably in the EX2 limit. Exchange due to unfolding reactions may press these limits, especially when measured under extreme conditions, e.g., with thermophilic proteins. Tests for EX2 behavior include the classical experiment for simple pH dependence,[14] the difficult and relatively noisy nuclear overhauser effect (NOE) correlation experiment,[16] and most easily the comparison of the rates measured for several NHs in a cooperative unfolding with their expected chemical rates.[5]

The effort to measure the slowest hydrogens of a protein in order to obtain the global unfolding free energy can be defeated by the long times necessary for exchange. For example, consider hydrogens that exchange by way of global unfolding in a protein that is stabilized by a free energy of 10 kcal/mol. The exchange time constant at pH 7 and 20° would be about 1 month, and it increases by 30-fold for each 2 kcal of further stabilization. The time required can be reduced and additional stability information can be obtained by performing HX experiments with added denaturant and/or at elevated temperature (below).

An Example

Stabilization free energy decreases with denaturant according to Eq. (6).

$$\Delta G_u(\text{den}) = \Delta G(0) - m[\text{den}] \tag{6}$$

The m value is the slope of a plot of the unfolding free energy (ΔG_u) against denaturant concentration. The m value proportions to the amount of buried protein

[14] A. Hvidt and S. O. Nielsen, *Adv. Protein Chem.* **21,** 287 (1966).
[15] S. W. Englander and N. R. Kallenbach, *Q. Rev. Biophys.* **16,** 521 (1984).
[16] H. Roder, G. Wagner, and K. Wuthrich, *Biochemistry* **24,** 7396 (1985).

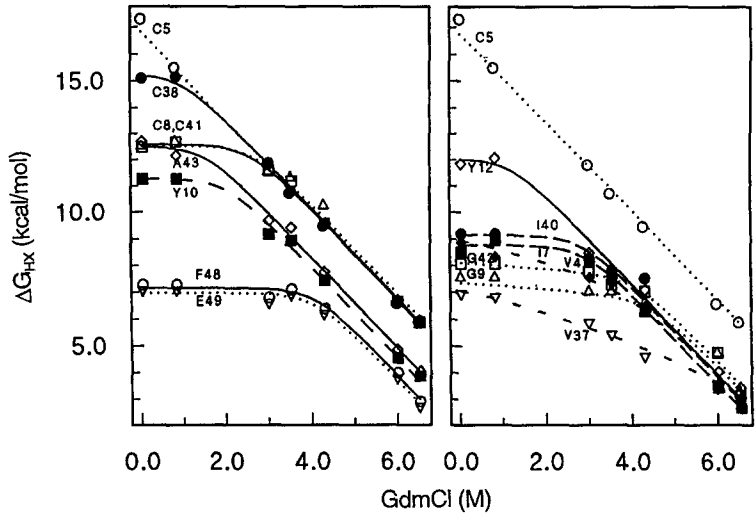

FIG. 2. HX results as a function of guanidinium chloride denaturant (GdmCl) for rubredoxin from *Pyrococcus furiosus*. Data were obtained by 1D NMR at 60° and computed using Eq. (3). The results distinguish HX that occurs by local fluctuations, a subglobal unfolding, and the highest free energy global unfolding. Reprinted with permission from R. Hiller, Z. H. Zhou, M. W. W. Adams, and S. W. Englander, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 11329 (1997).

surface that is exposed to solvent in the unfolding reaction. An example is given in Fig. 2, which shows HX results as a function of guanidinium chloride denaturant (GdmCl), measured by 1D NMR at 60° for rubredoxin from *Pyrococcus furiosus* and computed as in Eq. (3).

The data in Fig. 2 show that various amide hydrogens exchange at low denaturant with essentially zero m value, therefore by way of small H-bond breaking fluctuations that expose little new surface. Increasing denaturant selectively promotes larger unfolding reactions (decreases ΔG_u). A large unfolding can then come to dominate the exchange of the hydrogens that it exposes [Eq. (6)]. This is seen as the merging of local fluctuation curves into an unfolding curve. The highest free energy global unfolding of hyperthermophilic rubredoxin, with ΔG_u of 17 kcal/mol at 60° and zero GdmCl, involves the disruption of four Cys NH hydrogen bonds that group at the crown of the molecule where a functional metal atom is held. The bulk of the protein unfolds with the somewhat lower free energy of 14 kcal/mol (zero GdmCl, 60°).

HX rate is also accelerated by increased temperature. The intrinsic k_{ch} increases about 10-fold for each 20° to 25° rise in addition to the effect of temperature on K_{op}. Figure 3 shows temperature-dependent results for rubredoxin. NMR experiments up to 120° maintained exchange in the EX2 regime so that the stabilization free energy could be computed.

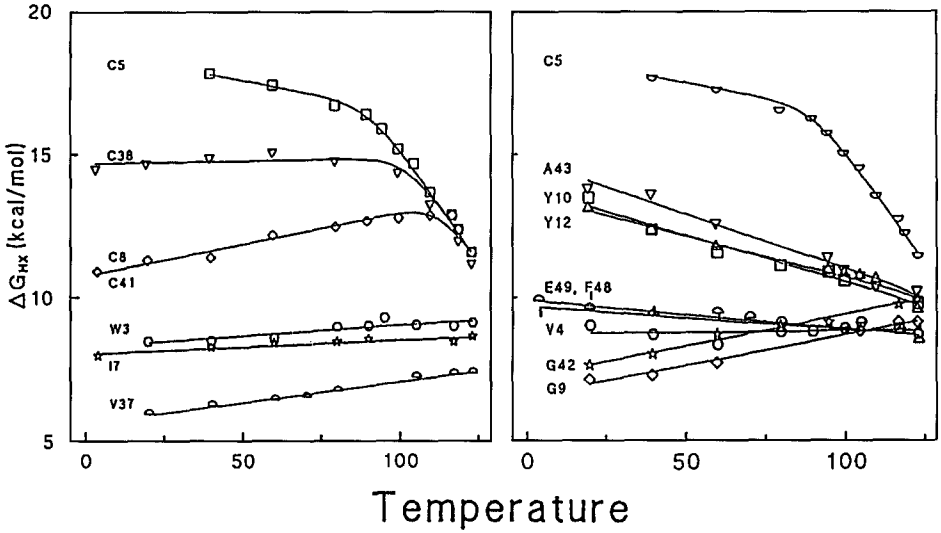

FIG. 3. HX results as a function of temperature for rubredoxin from *Pyrococcus furiosus*. Reprinted with permission from R. Hiller, Z. H. Zhou, M. W. W. Adams, and S. W. Englander, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 11329 (1997).

The slope of the temperature-dependent curve gives the entropy for the determining unfolding reaction ($d\Delta G/dT = -\Delta S$). From ΔG and ΔS, the enthalpy of the unfolding reaction can be directly computed ($\Delta G = \Delta H - T\Delta S$). The melting temperature can be estimated by extrapolating the plot of ΔG vs T to zero ΔG (see Fig. 4). Thus HX measured as a function of temperature well below the T_m can provide all of the usual thermodynamic parameters. In addition, the denaturant dependence gives the structural parameter, m.

The behavior of the global unfolding reaction, taken from the Cys-5 behavior in Fig. 3, is shown in Fig. 4 and compared to two unusually stable mesophilic proteins of almost identical size. The hyperthermophilic rubredoxin attains an extraordinarily high melting temperature, close to 200°. The comparison in Fig. 4 shows that this is accomplished by an increase in unfolding free energy and perhaps also by a shift of the stability curve to higher temperature. The latter point, however, is somewhat ambiguous because of the difficulty of determining the true maximum of the stability curve.

Conformational Flexibility

The relationship of protein flexibility to stability and function has a long history.[17–19] The operational meaning of this term is ambiguous and its measurement is uncertain. How does measured "dynamics" relate to "flexibility"? Various aspects of the dynamic character of protein structure have been approached by

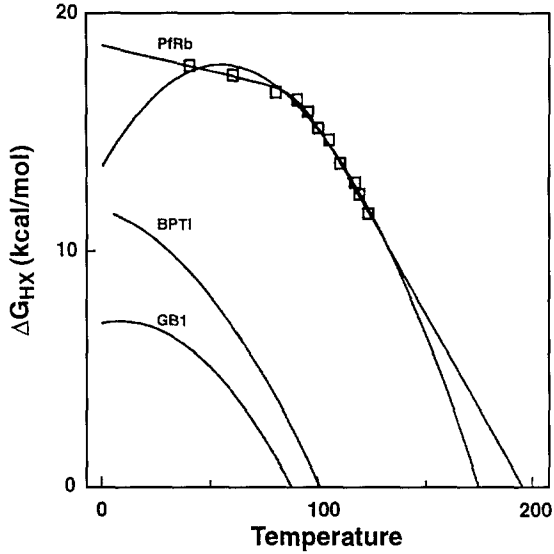

FIG. 4. Free energy of the global unfolding reaction, taken from the Cys-5 behavior in Fig. 3, compared to the analogous stability curves for two other unusually stable mesophilic proteins of almost identical size. The different curves through the rubredoxin (Rb) data indicate the uncertainty of extrapolation. The comparison can be viewed in the light of the type curves in Fig. 1. Reprinted with permission from R. Hiller, Z. H. Zhou, M. W. W. Adams, and S. W. Englander, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 11329 (1997).

X-ray diffraction, nuclear magnetic resonance, and theoretical methods, as well as by hydrogen exchange. It seems likely that these different approaches access different motions that occur on very different time scales.

Thermophilic proteins may provide a good testing ground for the functional significance of protein flexibility and dynamics. The Petsko laboratory has studied a thermophilic enzyme that essentially loses its activity at lower temperature where its closely related mesophilic counterpart is maximally active.[20,21] They present data that suggests some general relationship between the flexibility or rigidity of a protein, measured by HX, and its functional temperature range.[22]

HX measurements access two kinds of dynamically occurring distortions that are much smaller than the global unfolding discussed above. Very local fluctuations

[17] K. U. Linderstrom-Lang and J. A. Schellman, *in* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrback, eds.), pp. 443–510. Academic Press, New York, 1959.
[18] D. E. Koshland, G. Nemethy, and D. Filmer, *Biochemistry* **5,** 365 (1966).
[19] J. Monod, J. Wyman, and J. P. Changeaux, *J. Mol. Biol.* **12,** 88 (1965).
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[21] K. Hecht, A. Wrba, and R. Jaenicke, *Eur. J. Biochem.* **183,** 69 (1989).
[22] P. Zavodsky, J. Kardos, A. Svingor, and G. A. Petsko, *Proc. Natl. Acad. Sci. USA.* **95,** 7406 (1998).

can sever as few as one hydrogen bond at a time. The detailed motions involved are unknown and may represent, for example, some main chain twisting or bending that allows H-bond breakage and solvent access. It appears that the transient fluctuation necessary to bring an exchangeable hydrogen into fruitful H-bonding contact with solvent catalyst may require a fairly large separation of the protecting H-bond, perhaps by 4 Å or more.[23] Some hydrogens exchange by more extensive unfolding reactions involving entire secondary structural units, separately or together, mounting up to ultimate global unfolding.[24–28]

In applying HX methods to the flexibility problem, it will be necessary to distinguish the kind of underlying motions at work. Figures 2 and 3 show that the different kinds of fluctuations can be distinguished by their differing sensitivities to increasing denaturant and temperature. The slope of the free energy vs denaturant curve proportions to the amount of surface that is exposed in the opening reaction, indexed by the *m* parameter in Eq. (6). The slope of the free energy vs temperature curve gives the entropy of the responsible opening reaction. These parameters give information on the size of the structural distortion being measured in any particular case. In addition, it has been observed that neighboring hydrogens that exchange by way of small fluctuations by these criteria tend to exchange at very different rates. Neighboring hydrogens that appear to exchange by larger unfoldings should exchange with the same computed opening free energy, i.e., by way of the same larger scale opening reaction.

Summary

The naturally occurring hydrogen exchange of protein molecules can provide nonperturbing site-resolved measurements of protein stability and flexibility and changes therein. The measurement and understanding of these issues is especially pertinent to studies of thermophilic proteins. This chapter briefly reviews the considerations necessary for measuring hydrogen exchange and translating HX measurements into these detailed protein parameters.

Acknowledgment

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[25] A. K. Chamberlain, T. M. Handel, and S. Marqusee, *Nature Struct. Biol.* **3,** 782 (1996).
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[28] R. Hiller, Z. H. Zhou, M. W. W. Adams, and S. W. Englander, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 11329 (1997).